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# Dendritic cell immunotherapy in uterine cancer

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**Abbreviations:** AICD, activation-induced cell death**;** CCR, C-C chemokine receptor**;** CCL, chemokine ligand**;** CD, cluster of differentiation**;** CD40L, cluster of differentiation 40 ligand**;** DC, dendritic cell**;** DC-LAMP, dendritic cell lysosomal-associated membrane protein; FIGO, international ferderation of gynecology and obstetrics; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human lucocyte antigen; hTERT, human telomerase reverse transcriptase; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemotactic protein 1; MDSC, myeloid derived suppressor cell; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; mRNA, messenger ribonuclear acid; MUC1, mucin-1; RNA, ribonuclear acid; TAA, tumor-associated antigen; TAM, tumor-associated macrophage; TGFβ, tumor growth factor beta; TIL, tumor infiltrating lymphocyte; TLR, toll-like receptor; Treg, regulatory T cell; TNFα, tumor necrosis factor alfa; WT1, Wilms' tumor gene 1

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Uterine cancer is the most common pelvic gynecological malignancy. Uterine sarcomas and relapsed uterine carcinomas have limited treatment options. The search for new therapies is urgent. Dendritic cell (DC) immunotherapy holds much promise, though has been poorly explored in uterine cancer. This commentary gives an insight in existing DC immunotherapy studies in uterine cancer and summarizes

the possibilities and the importance of the loading of tumor antigens onto DC and their subsequent maturation. However, the sole application of DC immunotherapy to target uterine cancer will be insufficient because of tumor-induced immunosuppression, which willhamper the establishment of an effective anti-tumor immune response. The authors give an overview on the limited existing immunosuppressive data and propose a novel approach on DC immunotherapy in uterine cancer.

## Introduction

Worldwide, uterine cancer is the seventh most common malignant disorder and the most frequent pelvic gynecological cancer. Each year, in Europe, an estimated 9000 women die of the disease. It occurs mainly in postmenopausal women with a mean age of 65 y.

Uterine tumors can be epithelial or mesenchymal in origin and are designated as carcinoma and sarcoma, respectively. While sarcomas are uncommon but most of the time highly aggressive, uterine carcinomas comprise the majority of cancer cases of the corpus uteri, but normally have a lower grade malignancy potential.More than 73% of the patients with the most frequent subtype of endometrial cancer present at FIGO stage I, resulting in a 5-y survival of 85 to 90%. However, more rare subtypes of endometrial carcinoma as well as uterine sarcoma display a more aggressive behavior and thus often present in more advanced stages with a 5-y survival of 5–20%, depending on the subtype. Surgery is the cornerstone of treatment. The value of adjuvant chemotherapy is controversial for carcinoma and proven not beneficial for high grade uterine sarcoma. In advanced or recurrent disease, treatment options are strongly limited.[1,2]Therefore, there is an urgent need for exploration of new treatment options for uterine tumors.

One type of active immunotherapy is dendritic cell (DC) immunotherapy.DC have been discovered in 1973 by Ralph Steinman and since then, their role as sentinels of the

immune system that provide an essential link between innate and adaptive immunity has been elucidated. DC comprise only a minor (<1%) subset of the white blood cell population. Immature dendritic cells have the capacity to capture and process antigens, a process which leads to their maturation. Through interaction of the chemokine receptor CCR7 and its ligands CCL19/CCL21 DC migrate to the lymph nodes, where the processed antigens are presented to T cells, thus initiating an immune response.[3] Since the isolation of circulating DCs is challenging, DC immunotherapy is most frequently based on DCs cultured in an ex vivo setting starting from $CD34^+$ precursor cells or $CD14^+$monocytes. At the immature state DC are loaded with (defined) tumor antigens, offered as synthetic peptides or mRNA.

The application of active immunotherapy in the treatment of uterine cancer has not been explored for a long time and is still in its infancy, which is probably related to the poor insight into the interaction of the immune system with uterine cancers. However, as our knowledge increases, we believe that immunotherapeutic approaches hold promise for uterine cancer.Active immunotherapy relies on anticancer vaccines that are able to elicit an immune response against tumor-associated antigens (TAAs) in the human body. **Table 1** gives an overview of existing studies.

## Loading of Tumor Antigens

Immunotherapeutic targets can consist of one or more defined TAA or a tumor-derived mixture of unknown TAA. Several TAA are validated for immunotherapy in uterine tumors such as for example several cancer testis antigens,[8] MUC1,[9] universal TAA such as hTERT or antigens targeting tumor cell survival such as survivin (Vanderstraeten et al., unpublished data). Until now the majority of antigen-focused immunotherapeutic studies in general have used a single antigen as a target. However, more and more arguments are arising for settings using a combination of multiple antigens or the use of total tumor cell approach. There are 3 major advantages. First of all, by using a combination of several antigens, the risk

of immune escape is reduced. Second, by using multiple antigens, the group of tumors and hence the patient group that can be targeted with one treatment approach is much larger. Lastly, by including anti-apoptosis factors as a target, the survival of the tumor itself can be attacked as well. Nevertheless, the use of a total tumor cell approach (tumor lysate, total tumoral mRNA,…) can theoretically induce auto-immunity, even though a certain amount of autoimmunity is necessary for efficient tumor eradication. To date, no reports of this type of side-effects have been published in the context of uterine cancer.

A crucial component in any DC vaccine is the choice of the DC loading strategy. Immunotherapy usingDC loaded with peptide(s)is easily feasiblebut has several disadvantages, such as HLA restriction, the lack of a broad repertoire of MHC class II binding peptides and a short duration of antigen presentation. The in vitro work of several research groupshas suggested that RNA transfection of TAA is an effective, if not superior, method to generate immunostimulatory DCs. By using mRNA encoding the whole TAA, RNA transfectionis independent of the patients HLA-type.[10,11]In addition, further modification of the coding sequence by adding lysosomal targeting sequences (e.g., DC-LAMP) leads to the presentation of antigenic peptides in the context of both HLA class I and class II.[12-14]

Our research group has proven the safety and feasibility of DC immunotherapy in large series of high-grade glioblastoma (DCs loaded with lysate)[15] and in case reports of uterine and ovarian tumors (*WT1* mRNA-loaded DCs).[5,6,16]

## Maturation Status of DC

DC vaccines should be able to induce high avidity $CD8^+$ T cells that express high levels of granzyme and perforin and are able to enter the tumor microenvironment to exert their function. The quality of the induced $CD8^+$ T cells is influenced by the expression of co-stimulatory molecules, such as CD80, CD86, CD137, and CD252 on DC, the cytokine secretion pattern of DC and the type of $CD4^+$ helper T cell response.[17-19]DC maturation is one

of the crucial factors.[20,21] Currently, the "golden standard" used to mature DC consists of a cocktail of pro-inflammatory cytokines (IL-1β, IL-6, TNFα) and prostaglandin $E_2$.[22]However, a whole scale of cytokines, all mimicking the in vivo danger signal, is available to mature DC, such as IRX-2and CD137.[23,24]Recent mouse data demonstrated, however, that maturation of DC solely by pro-inflammatory cytokines yielded DC that failed to efficiently direct effector T cell differentiation. Interestingly, DC matured in the presence of Toll like receptor (TLR) ligands were able to induce full T cell effector function and induce more potent immune responses.[25,26]

More recent in the field of DC maturation is the use of TriMixco-electroporation (caTLR4 [constitutively active Toll-like receptor 4], CD40L, and CD70 mRNA). The combination of CD40L and caTLR4 electroporation mimics CD40 ligation[27,28] and TLR4 signaling[29] of the DC and generates phenotypically mature, cytokine/chemokine-secreting DC, as has been shown for CD40 and TLR4 ligation through addition of soluble CD40L and lipopolysaccharide (LPS).[30] On the other hand, the introduction of CD70 into the DC provides a co-stimulatory signal to $CD27^+$ naive T cells by inhibiting activation-induced cell death (AICD) of T cells and by supporting T cell proliferation.[31]DC co-electroporated with TriMix and tumor antigens have been shown to be potent stimulators of anti-tumor immunity in melanoma patients.[32-35]Providing the DCs with a maturation signal through mRNA electroporation offers several advantages. There is no need to pre-incubate the DCs for up to 48 h with soluble maturation signals like pro-inflammatory cytokines or TLR ligands to achieve DC activation, which can render the cells "exhausted" and inferior for vaccination purposes.[36] As a result, TriMix DC, which can be injected into the patient within a short period after electroporation, will mature and secrete most of their immunostimulatory cytokines and chemokinesin situ. Furthermore, it has been postulated that maturation of DCs

in situ resembles more closely the physiologic process involved in response to pathogen infection and may therefore lead to enhanced T-cell immunity.[37]

## Tumor Microenvironment

The sole application of DC immunotherapy to target uterine cancer is, however, not sufficient because of tumor-induced immunosuppression. More and more attention is being brought to the infiltrationof immune suppressive cells in the tumor microenvironment as a response to classical treatments and immunotherapeutic strategies. These immune suppressive cells hamper the establishment of an effective anti-tumor immune response. To our knowledge, this has been poorly studied in uterine cancer and results are often contradictory.

The role of tumor-associated macrophages (TAMs) in endometrial carcinoma is complex. Located in close contact with cancer cells, they have a beneficial effect on relapse-free survival, but TAMs located in necrotic tumor tissue are linked with tumor recurrence.[38] Moreover, TAMs secrete matrix metalloproteinases and induce angiogenesis which promote tumor progression.[39]Furthermore, TAMs are associated with myometrial invasion and lymph node metastasis and thus have a clear tumor-promoting role.[40,41]

Endometrial tumors are frequently infiltrated by T lymphocytes as well. $CD8^+$ lymphocytes present in primary untreated stage IA-IIIA endometrial cancer, show upregulation of inhibitory natural killer receptors, an effect that possibly is mediated through TGF-β. However, this upregulation prevents the cytotoxic function of these lymphocytes at the HLA recognition level, thus causing an important tumor evasion strategy, since non-classical HLA molecules are upregulated in cancer.[42]A recent publication showed the association of uterine sarcoma with systemic inflammation, as measured by an enhanced neutrophil to lymphocyte ratio in these patients.[43]Tumor-infiltrating lymphocytes (TIL) can be detected in uterine sarcoma, but these cells can sometimes harbor chromosomal aberrations which might negatively impact their functionality.[44]

In many tumors, regulatory T cells (Treg) suppress endogenous and induced antitumor immune responses. However, in endometrial carcinoma, the presence of Treg is debatable. Fattorossiet al.[45] showed their presence in the tumor-draining lymph nodes of 26 patients, whereas Giatromanolakiet al.[46] demonstrated less Treg in 79 stage I endometrial carcinoma patients compared with normal endometrium.Recent work of both Yamagamiet al.[47] and Zhang et al.[48] demonstrated an increase of Treg in the tumor tissue and peripheral blood respectively in endometrial carcinoma, increasing in more advanced stages. In contrast, the group of Sawanet al.[49]attributed this increase in Tregs to an increase in age and to the postmenopausal condition of most patients.

More recently, myeloid derived suppressor cells (MDSC) have been shown to play an important role in cancer immunosuppression and have been implicated in several tumor types. We showed for the first time that both endometrial carcinoma and uterine sarcoma tumors contain MDSC infiltrates that express arginase-1.[50]

## Proof of Concept

In order to improve our previous DC vaccination regimen, several modifications were incorporated:

1) Inclusion of both WT1 and survivin as targets to broaden the immune response

2) Inclusion of the DC-LAMP targeting signal in the WT1 and survivin mRNA for induction of both $CD4^+$ and $CD8^+$ T cells

3) Co-electroporation of WT1 and survivin with TriMix to induce DC maturation.

The feasibility of this approach was tested on leukapheresis products of 3 healthy donors. Monocytes were isolated by elutriation and cultured in CellGenix culture bags in the presence of GM-CSF and IL-4 (Cellgenix) until day 7. Immature DC were harvested, electroporated with mRNA encoding WT1-DC.LAMP and TriMix or survivin-DC.LAMP and

TriMix, mixed and cultured for 3.5 to 4 h in the presence of rapamycin (MiltenyiBiotec Leiden), IL-4, and GM-CSFbefore cryopreservation.

Upon thawing, DC showed a viability of 57.3 ± 5.5% and a purity of 64.6 ± 7.8%. DC phenotype was analyzed after thawing (4h after electroporation) and after an additional 44h culture period (48h after electroporation). Immediately after thawing, DC still presented with a rather immature phenotype, but became fully mature at 48h after electroporation, with upregulation of CD54, CD80, CD83, CD86, and CCR7 (**Table 2**). WT1 expression by DC was at the highest level 1.5 h after thawing and steadily decreased thereafter, while survivin only slightly decreased at 48h after thawing (**Fig. 1**). DC secreted low levels of IL-12p70, intermediate levels of IL-1β and IL-18 and high levels of IL-6, IL-8, MCP-1, and MIP-1α. Upon stimulation of autologous T cells, we observed an increase of survivin-tetramer (survivin 95–104 peptide) positive T cells from almost undetectable (0.08% of $CD8^+$ T cells) to 0.56% of $CD8^+$ T cells after 1 stimulation (7-fold) and to 0.81% of $CD8^+$ T cells after 2 stimulations with DC (10-fold). No induction of WT1-specific T cells was noted (**Fig. 2**).

## Conclusions

DC immunotherapy is in its infancy in uterine cancer. Nevertheless, the recent data concerning the interaction of uterine tumors with the immune system indicate that immunotherapy is promising as a novel therapeutic approach for this type of cancer. Furthermore, immunotherapeutic approaches are associated with less toxicity and fewer side effects, which is important for an elderly patient population. We have previously shown the feasibility of DC vaccination in uterine cancer and we anticipate that our novel approach will further improve this modality. Moreover, in our opinion, the combination of DC vaccines with inhibitors of immunosuppression holds great promise for uterine cancer patients.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Figure 1.**WT1 or survivin antigen expression by mRNA electroporated DC. DC electroporated with WT1.DC-LAMP + TriMix or survivin.DC-LAMP + TriMix were analyzed at 4h or 48h after electroporation for expression of WT1 or survivin by immunocytochemistry. The percentage of stained cells (left panel) and the intensity of staining (right panel) was documented.

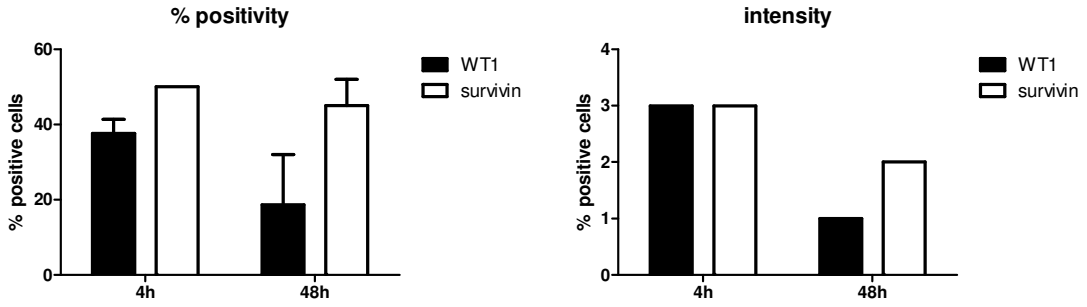

**Figure 2.**Tetramer analysis of stimulated T cells. DC electroporated with WT1.DC-LAMP + TriMix or survivin.DC-LAMP + TriMix were used at 4h post electroporation to stimulate autologous T cells. Unstimulated T cells and stimulated T cells were stained for CD3, CD8, and the respective tetramer. Cells were gated on FSC/SSC characteristics and $CD3^+$ $CD8^+$ cells. On the $CD3^+$ $CD8^+$ T cells, the percentage of WT1- or survivin-tetramer positive cells was determined.

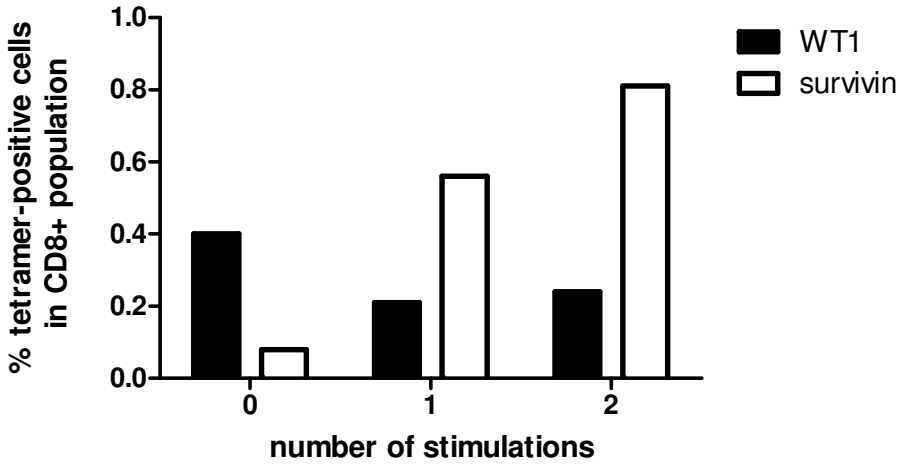


**Table 1.** Overview on DC immunotherapy in uterine cancer

| Group | Tumor | Number of Patients | Type of Active Immunotherapy |
|---|---|---|---|
| Santin A et al.[4] 2002 | Serous endometrial carcinoma | 3 | Injection of DCs loaded with whole tumor lysate |
| Hernando JJ et al.[5] 2002 | Uterine sarcoma | 2 | Injection of DCs loaded with whole tumoral lysate and KLH |
| Coosemans A et al.[6] 2010 | Serous endometrial carcinoma | 1 | WT1-mRNA loaded DC immunotherapy |
| Coosemans A et al.[7] 2013 | Leiomyosarcoma and serous endometrial carcinoma | 6 | WT1-mRNA loaded DC immunotherapy |

KLH, Keyhole Limpet Hemocyanin; WT1,Wilms' tumor gene 1; DC: dendritic cell.

**Table 2.** Characteristics of DC from 3 test leukaphereses (n = 3)

| Test | 4 hours | 48 hours |
|---|---|---|
| **Sterility** | | |
| bacteria | negative | negative |

| | | |
|---|---|---|
| mycoplasm | negative | negative |
| fungi | negative | negative |
| endotoxins | negative | negative |
| **mDC-identity*** | | |
| CD11c | 90.9% (12.7) | 95.4% (2.2) |
| CD14 | 11.8% (8.3) | 5.3% (5.3) |
| CD54 | 89.1% (11.8) | 98.0% (3.7) |
| CD80 | 38.1% (15.5) | 98.3% (1.0) |
| CD83 | 30.4% (8.0) | 65.4% (9.0) |
| CD86 | 76.7% (18.4) | 98.3% (0.6) |
| CCR7 | 25.8% (11.4) | 96.5% (2.0) |
| HLA-ABC | 99.0% (0.6) | 99.7% (0.2) |
| HLA-DR | 98.1% (2.2) | 97.6% (2.1) |
| **mRNA electroporation*** | | |
| CD70 | 45.5% (11.8) | 57.9% (22.8) |
| **Viability upon thawing*** | | |
| | 57.3% (5.5) | 57.3% (5.5) |
| **Purity upon thawing*** | | |
| | 64.6% (7.8) | 64.6% (7.8) |

*Results expressed as mean percentage (standard deviation).